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# **PROTEOLYSIS OF INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-1, -2 AND -4**

## **FUNCTION OF FRAGMENTS**

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*To Daniel, Anton and Oliver*

## ABSTRACT

The insulin-like growth factors (IGF-I and IGF-II) stimulate cell growth, survival and differentiation, and have insulin-like activity. A family of six IGF-binding proteins (IGFBP-1 through -6) bind to IGFs with high affinity and modulate their activity in the circulation and locally at cell-surface receptors. IGFBPs can potentiate or inhibit IGF-activity. Proteolysis is recognised as the predominant mechanism for IGF release from IGFBPs. IGFBPs have also IGF-independent effects, through the interaction with cell surface structures and extracellular molecules. IGFBP-1 and IGFBP-2 have a C-terminal Arg-Gly-Asp (RGD) sequence, and IGFBP-1 has been shown to increase migration independently of IGF-I by binding to extracellular  $\alpha 5 \beta 1$ -integrins. An IGFBP-1- protease activity has previously been isolated from the urine of a patient with multiple myeloma and an inflammatory skin disease. Although it was not isolated in its active form, the protease-activity was identified as azurocidin. The aim of this thesis was to further characterize this protease-activity and to study the biological effects of the proteolytic IGFBP fragments on migration and on IGF-stimulated proliferation in human dermal fibroblasts.

Intact IGFBP-1 and IGFBP-1 fragments, obtained by incubating with the patient-derived protease-activity, were characterized in four RIAs and with SDS-PAGE. Immunoreactivity and size of fragments were compared to in vitro produced fragments. Serum from the patient inhibited IGFBP-1 protease activity; however, immunoreactive IGFBP-1 in patient serum was present at molecular masses consistent with IGFBP-1 fragments, in addition to intact IGFBP-1. We also studied a neutrophil-derived preparation of azurocidin and found that it cleaved IGFBP-1, IGFBP-2 and IGFBP-4. IGFBP-1 bound to IGF-I was also degraded whereas IGF-II was shown to have an inhibitory effect on proteolysis of IGFBP-1. The proteolytically active preparation of neutrophil-derived azurocidin was found to be glycosylated and determined to be 31 kDa by SDS-PAGE. The same cleavage pattern of IGFBP-1 was obtained by both azurocidin-preparations derived from either urine or neutrophils. IGFBP-1, IGFBP-2 and their proteolytic fragments stimulated migration of fibroblasts and the stimulatory effect was abolished by pre-treating cells with an  $\alpha 5 \beta 1$  integrin antibody. High glucose impaired migration. However, the addition of IGFBP-1, IGFBP-2 or fragments increased migration to normal levels again. IGFBP-2 inhibited IGF-II induced proliferation, while IGFBP-2 fragments had reduced inhibitory effect. Intact phosphorylated IGFBP-1 showed either potentiating or inhibitory effects on IGF-I induced proliferation depending on the confluence of cells, and proteolysis of IGFBP-1 did not change these effects.

In conclusion, a novel IGFBP-protease which is associated to inflammation regulates IGF-activity in tissue through cleavage of IGFBPs, resulting in proteolytic IGFBP-fragments with biological effects important for tissue repair.

**Keywords:** Insulin-like growth factor, IGF-binding protein-1, IGF-binding protein-2 IGF-binding protein-4, IGFBP protease, azurocidin/heparin binding protein/CAP37, inflammation, migration, proliferation, high glucose

## LIST OF PUBLICATIONS

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## LIST OF ABBREVIATIONS

ALS	Acid-labile subunit
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BPTI	Bovine pancreatic trypsin inhibitor
CAP37	Cationic antimicrobial protein of 37 kDa
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle Medium
DSS	Disuccinimidyl suberate
ECL	Electrochemiluminescence
ECM	Extracellular matrix
FCS	Fetal calf serum
FPLC	Fast protein liquid chromatography
FSH	Follicle-stimulating hormone
GAG	Glycosaminoglycan
GH	Growth hormone
HBP	Heparin-binding protein
HBD	Heparin-binding domain
HepG2	Human-hepatoma-derived cell line
HMC-1	Human mast cell-1 line
HPLC	High performance liquid chromatography
ICAM-1	Intracellular adhesion molecule 1
IGF	Insulin-like growth factor
IGF-1R	Type 1 insulin-like growth factor receptor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
kDa	Kilo Dalton
LID	liver-specific igf-1 gene deleted
LPS	Lipopolysaccharide
L-domain	Linker-domain
MALDI/TOF MS	Matrix-assisted laser desorption/ionization/time of flight mass spectrometry



MAP	Mitogen-activated protein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NLS	Nuclear localization signal
PAPP-A	Pregnancy-associated plasma protein-A
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM-1	Platelet/endothelial cell adhesion molecule
PI3-kinase	Phosphatidylinositol-3-kinase
PMN	Polymorphonuclear leukocytes
PNGase F	Peptide N Glycosidase F
PPP	Picropodophyllin
PTH	Parathyroid hormone
recIGF-I	Recombinant insulin-like growth factor I
RGD	Arg-Gly-Asp (arginine-glycine-aspartic acid)
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SMC	Smooth muscle cells
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TA	Thymine adenine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
TNF- $\alpha$	Tumour necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule 1



# 1 INTRODUCTION

## 1.1 THE INSULIN-LIKE GROWTH FACTORS

The insulin-like growth factors (IGF-I and IGF-II) are small homologues peptides of approximately 7 kDa, which are structurally similar to insulin. They are expressed in most organs and promote cell growth, survival and differentiation, and have insulin-like activity (Enberg et al., 1984; Jones and Clemmons, 1995). IGFs function in an endocrine fashion as a hormone and locally as autocrine/paracrine factors. Growth factors are essential for normal growth and development.

### 1.1.1 IGF-receptors

The biological actions of IGFs are primarily mediated through the type 1 IGF receptor (IGF-1R) located at the cell surface (Furlanetto et al., 1987; Jones and Clemmons, 1995; Le Roith et al., 2001a). This receptor belongs to the tyrosine kinase receptor family and ligand binding induces IGF-1R auto-phosphorylation. The activated IGF-1R can then activate multiple signal transduction cascades, including the mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol-3-kinase (PI3-kinase)-Akt pathway (Duan and Xu, 2005). The IGF-1R receptor has a high degree of homology with the insulin receptor, which IGFs also can bind to, although with lower affinity compared with the IGF-1R. IGFs can exert insulin-like activity in tissues expressing high levels of IGF-1R (skeletal muscle, pancreatic  $\beta$ -cells (Silha, 2005). As a result of the close homology between IGF-1R and insulin receptors there are also hybrid IGF/insulin receptors, which bind to IGFs and insulin with similar affinities as the IGF-1R. The IGF-1R has 2- to 15-fold lower affinity for IGF-II compared with IGF-I (Jones and Clemmons, 1995; Le Roith et al., 2001a).

The IGF-II receptors have high affinity for IGF-II, however, there is no known IGF signalling mediated through this receptor. Binding of IGF-II to this receptor causes internalization and degradation of IGF-II and thereby reduces the levels of IGF-II, which is its primarily responsibility during fetal development. The IGF-II receptors are identical to the cation-independent mannose 6-phosphate receptors which function in the trafficking of lysosomal enzymes (Le Roith et al., 2001a).

### **1.1.2 Regulation of IGFs**

Growth hormone (GH) stimulates the production of hepatic IGF-I, which is the major source of circulating IGFs (Le Roith et al., 2001a). GH also induces IGF-I expression in many tissues e.g. liver, muscle, pancreas, intestine, kidney, adipose tissue and brain (Roberts et al., 1987). IGF-I was previously known as somatomedin and according to the original “somatomedin hypothesis”, the effects of GH on postnatal growth were mediated by IGF-I, which was secreted into the circulation from the liver. However, it was later discovered that IGF-I was produced locally in most tissues. The hypothesis was therefore revised to include an autocrine/paracrine function of IGF-I, which also was believed to be regulated by GH. Later studies have shown that there are other factors, besides GH, that can stimulate expression of IGF-I (Le Roith et al., 2001b). For example estrogen induces mRNA expression of IGF-I in the uterus, follicle-stimulating hormone (FSH) stimulates expression in the ovary and parathyroid hormone (PTH) and estrogen increase expression in bone (Hatey et al., 1992; Murphy et al., 1987). Nutrition is one of the major regulators of IGF-I in the circulation. IGF-I has anabolic effect on protein metabolism and is reduced by food deprivation and in catabolic stress (J.P Thissen, 2005).

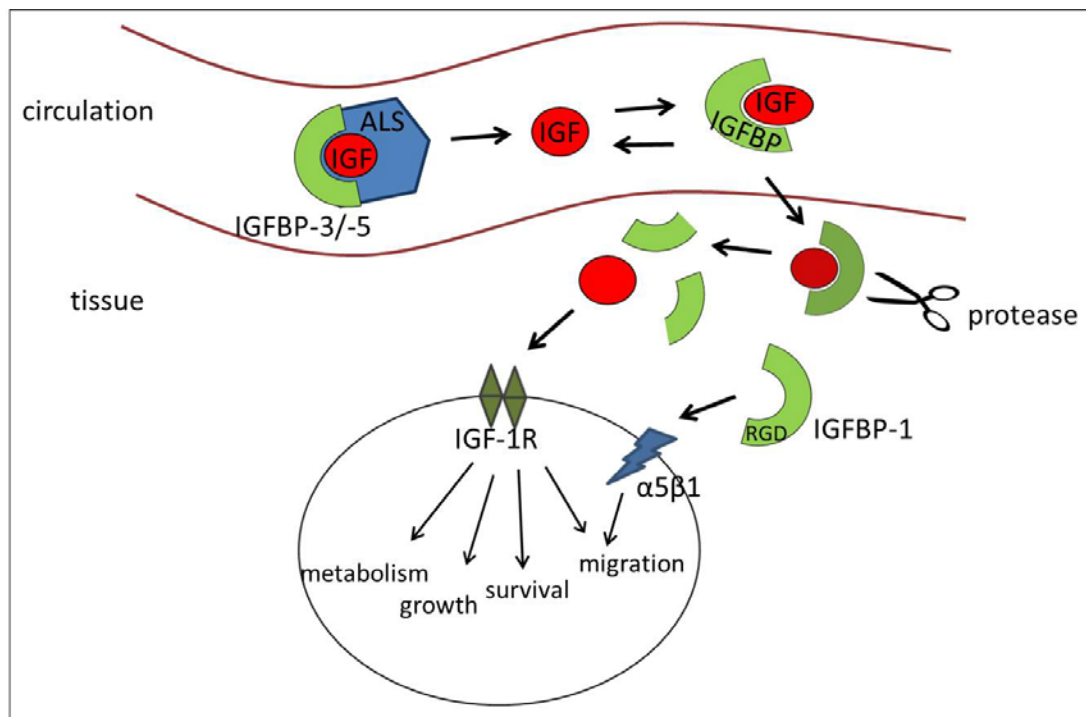
The liver-specific *igf-1* gene deleted mouse (LID mouse) model has revealed that liver-derived IGF-I is not essential for postnatal growth (Sjogren et al., 1999; Yakar et al., 1999). However, subcutaneous IGF-I therapy resulted in significant growth response in patients with GH receptor dysfunction or IGF-I gene deletion, implicating the importance of endocrine IGF-I for human growth (Azcona et al., 1999; Camacho-Hubner et al., 1999; Ranke et al., 1999). Liver-derived IGF-I has also been shown to be involved in the regulation of blood pressure in LID mice (Tivesten et al., 2002). Inflammatory cytokines can act systemically to impair IGF-I synthesis but also to affect growth locally. IL-1b and TNF-alpha inhibited longitudinal growth in fetal rat metatarsal bones which was partly reversed by IGF-1 (Martensson et al., 2004; Schmeling et al., 2003).

## **1.2 IGF-BINDING PROTEINS**

The activity of IGF-I and IGF-II is regulated by a family of six IGF binding-proteins (IGFBPs), which bind to IGFs with high affinity. These binding proteins serve as carriers of IGFs, prolong IGF half-life in the circulation and modulate IGF activity at cell-surface receptors (Firth and Baxter, 2002a; Jones and Clemmons, 1995). IGFs in

the circulation are mainly transported in a 150 kDa ternary complex with IGFBP-3 and the acid-labile subunit (ALS). The ternary complex is too large to cross the endothelial cell layer and prolongs the very short half-lives of IGFs in the circulation by approximately 12-15 h. The complex is considered to be the storage pool of IGFs and IGFs are released from this 150 kDa-complex principally by limited proteolysis of IGFBP-3 (Jones and Clemmons, 1995; Rechler and Clemmons, 1998). All members of the ternary complex are induced by GH and it is assumed that the complex formation occurs in the liver since IGF-I and ALS are produced by hepatocytes, whereas IGFBP-3 is derived from Kupffer cells (Arany et al., 1994; Chin et al., 1994). IGFBP-5 has also been shown to be part of a ternary complex, although to a much lesser extent (Firth and Baxter, 2002a).

IGFs also exist in smaller binary complexes (40-50 kDa) together with IGFBPs. The binary complexes have the ability to cross the vascular wall and that makes them important IGF transporters to target tissues, where IGFs elicit hormonal growth-stimulating effects through the IGF-1R (Jones and Clemmons, 1995). Free IGFs disappear from the circulation within minutes (Hodgkinson et al., 1989) (**Figure 1**).



**Figure 1.** IGFBPs regulate the activity of IGFs in circulation and tissue.

IGFBPs are expressed in many peripheral tissues and each IGFBP has specific tissue production which results in tissue-specific actions of IGFs (Lee et al., 1997). IGFBP-4 and IGFBP-6 have been consistently found to inhibit IGF activity, while IGFBP-1, 2, -3 and -5 can inhibit or potentiate IGF actions in vitro (Duan and Xu, 2005; Mohan and Baylink, 2002). IGFBP-1 has earlier been shown to potentiate IGF-stimulated proliferation in cell cultures of human, mouse and chicken embryo fibroblasts (Elgin et al., 1987). Non-phosphorylated IGFBP-1 has been reported to be associated with potentiation of IGF-I activity, while phosphorylated IGFBP-1 has been reported to inhibit IGF action (Jyung et al., 1994). Pre-incubations of human skin fibroblasts with IGFBP-3 potentiated IGF-I stimulation, in contrast to co-incubation of IGF-I and IGFBP-3, which caused inhibition of proliferation (De Mellow and Baxter, 1988). IGFBP-5 bound to extracellular matrix (ECM) was found to potentiate IGF-I induced cell proliferation (Jones et al., 1993b).

### **1.2.1 IGFBP-1**

Povoa et al. isolated and characterized the first IGF-binding protein from mid-term amniotic fluid and developed a radioimmunoassay in our laboratory in 1984, which measured both phosphorylated and non-phosphorylated IGFBP-1 (Povoa et al., 1984a; Povoa et al., 1984b).

IGFBP-1 has a rapid turnover and a marked circadian rhythm with several-fold higher levels during night compared to in the afternoon (Brismar et al., 1988). The serum levels of IGFBP-1 are age dependent with the highest levels at birth and lowest during adolescence (Hall K., 1988). Endocrine IGFBP-1 is mainly produced in liver and is secreted in a highly phosphorylated form that has high IGF-I affinity (Lewitt, 1994). Unlike other IGFBPs, IGFBP-1 is acutely regulated in response to insulin and is likely to have an important endocrine function in nutrition by inhibiting the insulin-like activity of IGFs (Brismar et al., 1994; Brismar et al., 1988; Lee et al., 1997; Lewitt, 1994; Tivesten et al., 2002). IGF-I lower blood glucose levels directly by stimulating glucose uptake in cells and indirectly by increasing insulin sensitivity in tissues (Wheatcroft and Kearney, 2009). IGFBP-1 is increased during short-term fasting due to nutritional deprivation and in response to insulin-induced hypoglycaemia (Bang et al., 1994b; Lewitt, 1994). Circulating IGFBP-1 is predominantly regulated by insulin, which inhibits the expression of IGFBP-1 at the transcriptional level in hepatocytes (Brismar et al., 1994; Durham et al., 1999). Besides insulin and nutrition, IGFBP-1 is

also regulated by cytokines, GH, IGFs, hypoxia and stress hormones (Brismar et al., 1995; Hilding et al., 1993; Silha, 2005) (**Table 1**).

Tissue-specific gene expression of IGFBP-1 has also been detected in kidney, decidualized stromal cells of the uterine endometrium and in luteinizing ovarian granulosa cells (Lee et al., 1997). In conditions with inflammation, IGFBP-1 can be expressed for example in atherosclerotic plaque (Wang et al., 2011). A number of studies indicate a role of IGFBP-1 as a regulator of IGF bioavailability in embryonic growth. IGFBP-1 increases in maternal circulation, is the predominant IGFBP in amniotic fluid and a major IGF-binding protein in fetal plasma (Drop et al., 1984; Han et al., 1996; Kajimura et al., 2005). IGFBP-1 modulates the free fraction of IGFs in serum and can thus be used as a surrogate marker of free IGF-I (Frystyk et al., 1994).

**Table 1.** Regulators of IGFBP-1 and IGFBP-2

	IGFBP-1	IGFBP-2
Fasting	↑	↑
Amino acids	↓	
Nutrition		↓
Insulin	↓	↓
Cortisol	↑	↓
Catecholamines	↑	
Glucagon	↑	
GH	↓	↓
IGFs	↓	
Cytokines	↑	
Leptin	↓	↑
Hypoxia	↑	

### 1.2.2 IGFBP-2

IGFBP-2 is the second most abundant binding protein in the circulation and is predominantly expressed in adipocytes, liver, reproductive system and central nervous system (Shimasaki and Ling, 1991; Wheatcroft and Kearney, 2009). IGFBP-2 is the dominating IGFBP in adipose tissue, lymph, bronchoalveolar lavage, prostate and seminal fluid (Chadelat 1998, Rosenfeld 1990). In general, the function of IGFBP-2 is to inhibit IGF actions, in particular IGF-II, since it has a higher affinity for IGF-II than IGF-I (Firth and Baxter, 2002a). Little is known about post-translational modifications of IGFBP-2. Circulating IGFBP-2 is inhibited by insulin, however, less rapidly than

IGFBP-1. IGFBP-2 is also inhibited by GH and increased after extended fasting (**Table 1**). IGFBP-2 levels correlates inversely with body mass and insulin concentrations (Silha, 2005; Wheatcroft and Kearney, 2009). IGFBP-2 has been suggested as a marker for the metabolic syndrome, since these patients have lower IGFBP-2 concentrations (Heald et al., 2006).

### **1.2.3 IGFBP-4**

IGFBP-4 is widely expressed in different tissues, with the highest expression in liver. IGFBP-4 can be N-glycosylated and does not bind to cell surface (Rechler and Clemmons, 1998; Shimasaki et al., 1990). The regulation of IGFBP-4 is cell type specific (Silha, 2005). IGFBP-4 is a potent inhibitor of IGF action in a variety of cell types, including bone and vascular smooth muscle cells. The inhibitory effect of IGFBP-4 on IGF is regulated by proteolysis of IGFBP-4, which results in release of free IGFs (Firth and Baxter, 2002a).

### **1.2.4 Post-translational modifications of IGFBPs**

The six IGFBPs have a mass of 24-45 kDa and are organized into three domains of approximately equal size. The N- and C-terminal domains of IGFBPs are conserved and cysteine rich (Mohan and Baylink, 2002). The non-conserved linker-domain (L-domain) is susceptible to post-translational modifications such as glycosylation, phosphorylation and proteolysis (Firth and Baxter, 2002a).

The high affinity interactions between IGFs and IGFBPs prevent binding of IGF to IGF-1R. Since the affinity of IGFs for IGFBPs are higher than their affinity for the IGF-1R, IGFBPs must be modified by post-translational modifications in order to release IGFs. Three post-translational modifications have been shown to influence the IGF binding affinity of IGFBPs (Duan and Xu, 2005; Mohan and Baylink, 2002). See **Table 2** for biochemical properties of IGFBPs.

#### *1.2.4.1 Proteolysis of IGFBPs*

Proteolysis of IGFBPs reduces their inhibitory effect on IGF actions and occurs predominantly in the non-conserved mid-region of IGFBPs (Bunn and Fowlkes, 2003; Firth and Baxter, 2002a). Proteases which can cleave IGFBPs into fragments with reduced affinity for IGFs have been identified in a variety of biological fluids. All six



**Table 2.** Biochemical properties of IGFBPs

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
ALS binding			+		+	
Glycosylation			+	+	+	+
Heparin binding sequence		+	+		+	+
Cell surface/ECM binding		+	+		+	
RGD sequence	+	+				
Nuclear localization sequence			+		+	
Ser phosphorylation sites	+		+		+	
Size (kDa)	25	31	43-45	24	29	28-30

IGFBPs can be cleaved by various IGFBP proteases and proteolytic cleavage has been recognised as the predominant mechanism for IGF release from IGFBPs (Bunn and Fowlkes, 2003). Proteolysis of IGFBP-3 and IGFBP-5 sometimes results in fragments with retained affinity for IGFs. IGFBP-3 and IGFBP-5 fragments have also been shown to potentiate IGF activity. In contrast, the proteolytic fragments of IGFBP-2 and IGFBP-4 have significantly reduced affinities for IGFs (Jones and Clemmons, 1995).

The N-domain of IGFBPs contains the major IGF binding site, but C-domains also contribute to the ligand-binding capacity (Clemmons, 2001). The relative IGF-binding affinities of isolated N- and C-domains differ among IGFBPs. The N- and C-terminal domains of IGFBP-2 cooperate in IGF binding and are both required to form a high affinity complex (Carrick et al., 2001). The amino-terminal domain is required for rapid binding of IGFBP-2 to IGF, while the carboxyl-terminal has higher affinity and is required to maintain IGFBP-2 and IGF in a complex form. Co-incubation of the N- and C-domains of IGFBP-2 did not enhance IGF-binding (Carrick et al., 2001). In contrast, the N-domain of IGFBP-4, -5 and -6 has higher affinity for IGF compared to the C-domain (Bach et al., 2005; Kalus et al., 1998; Standker et al., 2000).

#### 1.2.4.2 Phosphorylation of IGFBPs

IGFBP-1, IGFBP-3 and IGFBP-5 can be serine phosphorylated (Coverley and Baxter, 1997). IGFBP-1 is secreted by liver in a highly phosphorylated form that has high IGF-I affinity (Lewitt, 1994). Serine residues 101, 119 and 169 on IGFBP-1 can be phosphorylated which leads to a 6-7 fold increase in affinity for IGF-I (Jones et al., 1993a). The phosphorylated form of IGFBP-1 was first reported to be resistant to proteolysis, however, Wang et al. recently showed that the phosphorylated form of IGFBP-1 was efficiently cleaved by a protease activity purified from urine (Wang et

al., 2006b) Phosphorylation of IGFBP-3 protects against degradation in vitro and inhibits cell surface binding (Coverley et al., 2000). However, phosphorylation of IGFBP-3 does not appear to influence IGF binding affinity (Coverley and Baxter, 1997).

#### *1.2.4.3 Binding of IGFBPs to extracellular matrix and cell surface*

IGFBP-3 and IGFBP-5 can bind to the cell surface or extracellular matrix which has been shown to reduce their affinities for IGF-I (Mohan and Baylink, 2002). Binding of IGFBP-3 to fibroblasts reduced its affinity for IGF-I compared to IGFBP-3 in solution (Conover and Powell, 1991), while binding of IGFBP-5 to ECM lowered its binding affinity for IGF-I (Jones et al., 1993b). IGFBP-5 can bind to glycosaminoglycan (GAG) present in the ECM, which can result in cell association of IGFBPs and potentiation of IGF activity (Bach et al., 2005; Mohan and Baylink, 2002). IGFBP-5 associated to ECM is not cleaved by proteases, has reduced affinity for IGFs and potentiates IGF action (Firth and Baxter, 2002a).

Glycosylation of the non-conserved L-domain of IGFBPs affects stability, cell association, susceptibility to proteolysis and circulating half-life (Firth and Baxter, 2002a; Marinaro et al., 2000a; Marinaro et al., 2000b).

### **1.2.5 IGF-independent effects of IGFBPs**

IGFBPs also possess intrinsic IGF-independent effects on e.g. cell growth, differentiation and apoptosis. There have been reports of three groups of macromolecules which are capable of binding to IGFBPs, and possibly through which they could activate signalling pathways on their own (Ricort, 2004).

#### *1.2.5.1 Trans-membrane IGFBP receptors*

IGFBP-1 and IGFBP-2 contain a C-terminal Arg-Gly-Asp (RGD) sequence which can associate with cell-surface  $\alpha 5 \beta 1$ -integrin (fibronectin receptor) (Brewer et al., 1988; Jones et al., 1993c). Integrins are trans-membrane proteins which mediate interactions between the cytoskeleton and the extracellular matrix. The RGD sequence is also present in several extracellular matrix proteins such as fibronectin and vitronectin and is considered essential for cellular attachment of these proteins (Pierschbacher et al., 1982). Several studies have shown that IGFBP-1 increase migration independently of IGF-I by binding to  $\alpha 5 \beta 1$ -integrins (Gleeson et al., 2001; Jones et al., 1993c; Perks et al., 1999). For example IGFBP-1 stimulates human trophoblast migration by signalling

through  $\alpha 5\beta 1$ -integrin via MAP kinase pathway (Gleeson et al., 2001). There are only a few studies that have reported IGF-independent effects of IGFBP-2 through interactions of the C-terminal RGD sequence with  $\alpha 5\beta 1$ -integrins (Wheatcroft and Kearney, 2009). Binding of IGFBP-2 to the cell surface has been shown in the rat brain olfactory bulb (Russo et al., 1995) and Wang et al. has reported that IGFBP-2 induces migration through interaction with integrin  $\alpha 5$  and this was mediated through the RGD domain on IGFBP-2 (Wang et al., 2006a). IGFBP-5 has been shown to bind to an unidentified 420 kDa membrane protein in osteoblastic cells, which is followed by internalization of IGFBP-5 (Andress, 1995).

#### *1.2.5.2 Extracellular matrix receptors*

The C-terminal domains of IGFBP-3 and IGFBP-5 have heparin-binding domains (HBD) which are involved in most of the IGF-independent interactions of these binding-proteins (Firth and Baxter, 2002a). IGFBP-2 can interact with glycosaminoglycans (GAGs), but only in the presence of IGFs (Arai et al., 1996). IGFBP-4 has no HBD and has not been reported to interact with molecules on the cell surface (Firth and Baxter, 2002a)

#### *1.2.5.3 Intracellular IGFBP receptors*

Structural analyses of IGFBPs have revealed nuclear localization signals (NLS) in the C-terminal domain of IGFBP-3 and IGFBP-5 (Radulescu, 1994). There are several reports on IGFBP-3 located in the nucleus of cells (Lee and Cohen, 2002). IGFBP-5 has been shown to translocate to the nucleus in human breast cancer cells via the cytosolic IGFBP-5 “receptor” importin- $\beta$  (Schedlich et al., 2000; Schedlich et al., 1998).

Several IGFBPs have been reported to have both anti- and pro-apoptotic effects (Ricort, 2004). IGFBP-3 inhibits cell growth independently of IGFs and IGF-1R (Butt and Williams, 2001) and induces apoptosis in human prostate cancer cells (Hong et al., 2002). IGFBP-1 induces apoptosis in human breast cancer cells by interacting with integrin receptors (Perks et al., 1999).

IGFBP-fragments have also been reported to have IGF-independent effects. Sala et al. demonstrated that a C-terminal fragment isolated from amniotic fluid has IGF-independent effect on cell migration (Sala et al., 2005).

### 1.2.6 IGFBP proteases

IGFBP proteases have been identified in several body fluids and from conditioned media of various cell cultures (Conover, 1995; Rajaram et al., 1997). Protease inhibitors have been used to identify IGFBP proteases from several major classes of proteases; serine proteases, metalloproteinases, aspartic acid proteases and cysteine proteases. IGFBP proteases can be relatively specific or non-specific to a certain IGFBP.

Increased proteolysis of IGFBP-3 in serum has been observed during several pathological and physiological situations of increased growth like pregnancy and catabolic states. The pregnancy-associated IGFBP-3-specific protease was one of the first identified IGFBP proteases. This protease is specific to the reproductive tissues and releases IGFs in the second trimester of human to increase fetal growth (Giudice et al., 1990; Hossenlopp et al., 1990; Rajaram et al., 1997). An IGFBP-3 specific protease has been detected in the circulation during severe illness (Davies et al., 1991) and patients with acute and chronic renal failure had an increase of IGFBP-3-specific protease and absence of intact IGFBP-3 in urine (Lee et al., 1994). Increased levels of IGFBP-3 proteolysis has also been detected in patients with Type 2 diabetes (Bang et al., 1994a) and Type 1 diabetes in children (Bereket et al., 1995). It is believed that IGFBP-3 proteolysis occurs in catabolic conditions to increase bioactive IGFs.

Matrix metalloproteinases (MMP) secreted from human dermal fibroblasts has been reported to degrade IGFBP-3 and IGFBP-5 (Fowlkes et al., 1994; Nam et al., 1996). The lysosomal proteinase cathepsin D cleaves IGFBP 1-5, IGF-I and IGF-II under acidic conditions (Claussen et al., 1997; Conover and De Leon, 1994). It has been suggested that cathepsins participate in the clearance of IGFBPs and/or IGFBP-IGF complexes (Bunn and Fowlkes, 2003).

An IGFBP-4 specific protease was partially purified from human fibroblasts and identified as the pregnancy-associated plasma protein-A (PAPP-A). This protease was also found to be expressed by vascular smooth muscle cells, ovarian granulosa cells, osteoblasts and placental trophoblasts. Recent studies, however, indicate that IGFBP-2 and IGFBP-5 also may be substrates for PAPP-A (Boldt and Conover, 2007).

An IGFBP-5 protease was partially purified from human fibroblasts and identified as the serine protease complement protein 1s, which is part of the complement cascade (Busby et al., 2000).

The neutrophil-granular serine proteases elastase and cathepsin G, known as extracellular matrix degrading enzymes, have been reported to cleave all six IGFBPs

(Gibson and Cohen, 1999). A protease activity specific for IGFBP-1 was purified from the urine of a patient with multiple myeloma and an inflammatory skin disease (Wang et al., 2006b). This protease activity was identified as azurocidin, based on the presence of azurocidin in partially purified material from urine and the inhibitory effect of an antibody to azurocidin.

#### *1.2.6.1 Regulation of IGFBP proteases*

Proteolysis of IGFBPs is regulated by a variety of systemic and local mediators (Bang, 1995; Braulke et al., 1995; Clemmons et al., 1995; Collett-Solberg and Cohen, 1996; Conover, 1995; Fowlkes et al., 1995; Hughes et al., 1995; Rajah et al., 1995).

Insulin has been shown to regulate the IGFBP-3-specific activity as untreated Type 2 diabetes and Type 1 diabetes patients have increased serum levels of IGFBP-3 protease, which declines after insulin therapy (Rajaram et al., 1997).

Proteolysis of IGFBP-4 and IGFBP-2 by PAPP-A in cell-free assays have been shown to be IGF-dependent with IGF-II generally being more effective than IGF-I at stimulating proteolysis. Cleavage of IGFBP-5 by PAPP-A, however, is IGF-independent (Boldt and Conover, 2007). In contrast to PAPP-A, the proteolytic activity of a metalloproteinase secreted from human fibroblasts was inhibited by IGF-II (Bunn and Fowlkes, 2003). The proteolytic activity of a human fibroblast derived protease was decreased when IGFBP-5 was bound to ECM. Therefore it has been suggested that that ligand-induced conformational changes of IGFBPs can determine the ability of IGFBPs to be degraded by IGFBP proteases (Bunn and Fowlkes, 2003).

### **1.3 THE IGF-SYSTEM IN HEALTH AND DISEASE**

The IGF system is important in normal physiology and alterations are observed in pathological conditions. The expression of IGFs is age-dependent and normal levels range between 100-300 µg/L (Hilding et al., 1995). IGF-II predominates in the prenatal stage and is four times higher than IGF-I in human adults. IGF-I concentrations increase slowly in childhood and reaches the highest levels during puberty, and is then continuously decreased with age (Hilding et al., 1995; Juul, 2003; Lewitt, 2005).

Studies using the IGF-I and IGF-II knockout mice have shown that IGF-I and IGF-II are essential for fetal and postnatal growth, and survival. IGF-I knockout mice are born small and exhibit poor postnatal growth, if they survive at all (Liu et al., 1998; Liu et al., 1993). These animals are severely growth retarded and infertile. The IGF-II

knock-out mice had lower body weight at birth compared with the controls, however, they continued to grow postnatally at a rate similar to the wild-type mice. Although GH is not essential for prenatal growth, postnatal GH deficiency results in growth retardation (Gluckman et al., 1981). However, IGF-I infusion restores growth in GH-deficient mice (Guler et al., 1988).

Low levels of IGF-I are associated with Type 1 diabetes mellitus (T1DM) and IGF-I treatment improves glucose and protein metabolism and attenuates diabetic cardiomyopathy (Brismar et al., 1995; Camacho-Hubner et al., 1999; Carroll et al., 2000; Norby et al., 2002). The glycaemic control in type 2 diabetic patients was improved by IGF-I treatment (Moses et al., 1996) and skeletal muscle IGF-1R knock-outs resulted in insulin resistance and later development of type 2 diabetes mellitus (T2DM) (Fernandez et al., 2001; Lewitt et al., 2008; Petersson et al., 2009). Low IGFBP-1 levels are associated with insulin resistance and with cardiovascular disease risk, while decreased concentrations of IGFBP-2 are associated with obesity and has been suggested to be a marker for the metabolic syndrome (Heald et al., 2006; Wheatcroft and Kearney, 2009).

Epidemiological studies have shown that increased levels of IGF-I is a risk factor for the development of breast, prostate, colon and lung cancer. Besides, abnormally high levels of IGFs and/or IGF-1R have been detected in a number of tumour cells (LeRoith and Roberts, 2003). IGF-II is often up-regulated in cancer and has been proposed to be an autocrine regulator in tumor cells (Sciacca et al., 1999). In early breast cancer circulating free IGFs was shown to be elevated, while total IGF-II was decreased (Espelund et al., 2008).

Brain injury induces expression of IGF-I and IGF-1R and exogenous IGF-I treatment improved damages (Guan et al., 2003). IGF-I treatment have also been shown to have positive effects on Alzheimer disease and ALS (Carro et al., 2002; Kaspar et al., 2003).

## **1.4 AZUROCIDIN – AN INFLAMMATORY MEDIATOR**

In 1984 Shafer et al purified an antimicrobial protein of 37 kDa from the granules of human neutrophils and named it cationic antimicrobial protein of 37 kDa (CAP37) (Shafer et al., 1984b)(Shafer, Martin et al. 1984)(Shafer, Martin et al. 1984)(Shafer, Martin et al. 1984)(Shafer, Martin et al. 1984) (Shafer, Martin et al. 1984). Wilde et al. isolated azurocidin from the azurophilic granules of human neutrophils (Wilde et al., 1990b) and Flodgaard et al. isolated heparin-binding protein (HBP) by heparin affinity chromatography from human neutrophils (Flodgaard et al., 1991b). CAP37, HBP and azurocidin are three different names for the same protein and we will refer to this protein as azurocidin.

Azurocidin belongs to the serprocidin family of serine protease homologues (Flodgaard et al., 1991a) and is closely structurally related to three other neutrophil granule proteins; elastase, proteinase 3 and cathepsin G (Almeida et al., 1991b; Morgan et al., 1991a). Azurocidin has been considered proteolytically inactive due to the replacement of serine and histidine in the serine protease catalytic triad (Campanelli et al., 1990a; Pereira et al., 1990a; Wilde et al., 1990a). However, the inactive “catalytic site” of human azurocidin can still bind to the bovine pancreatic trypsin inhibitor (BPTI) or aprotinin (Petersen et al., 1993) and BPTI-affinity chromatography has been shown to be a very efficient method to purify azurocidin (Watorek et al., 1996).

### **1.4.1 Structure and biosynthesis of azurocidin**

Azurocidin is constitutively expressed during the promyelocyte stage of neutrophil differentiation and the proproteins are stored in the neutrophil granules (Gullberg et al., 1999).

Azurocidin is synthesized as a 251 amino-acid precursor, which is processed by removal of 26 amino acids from the N-terminus and three residues from the C-terminal (Almeida et al., 1991a; Morgan et al., 1991b). The mature protein is a single polypeptide, which consists of 222 amino-acid residues and has a calculated mass of 24 kDa (Pohl et al., 1990). Three N-linked glycosylation sites have been identified located at asparagine residues 100, 114 and 145 and the differences in the reported molecular masses (29–37 kDa) are probably due to different degrees of glycosylation (Watorek, 2003). Recombinant non-glycosylated azurocidin has been shown to have lower biological activity compared to glycosylated azurocidin, which suggests that glycosylation of azurocidin affects its biological activity (Iversen et al., 1999).

### 1.4.2 Biological activities

Azurocidin is a multifunctional protein with important functions in host defence and inflammation (Pereira, 1995).

#### 1.4.2.1 Antimicrobial activity

Azurocidin was first recognized for its bactericidal activity preferentially towards gram-negative bacteria, particularly *Salmonella typhimurium* and *Escherichia coli* (Shafer et al., 1984b; Shafer et al., 1986). The antimicrobial activity of azurocidin depends on ionic strength and pH and demonstrates activity in the concentration range of  $10^{-5}$ - $10^{-6}$  (Gabay et al., 1989; Shafer et al., 1986; Spitznagel, 1990). Pereira et al. have produced synthetic peptides to determine which part of azurocidin is important for the antibacterial activity and found that residues 20-44 of azurocidin has the highest bacterial activity, however, 10-fold lower than intact azurocidin (Pereira et al., 1993). Maximum activity of the peptide was determined at pH 5.0 – 5.5, and the activity was decreased at pH 7.0. This peptide also binds to lipopolysaccharide (LPS) and lipid A component of LPS, and has been shown to inactivate LPS-induced responses (Brackett et al., 1997; Pereira et al., 1993). LPS, also known as endotoxin, is the major component of the outer membrane of gram-negative bacteria.

Azurocidin might be a valuable tool to determine sepsis at an early stage, since azurocidin increases in sepsis and the levels correlate with the development of hypotension and circulatory failure (Linder et al., 2009).

#### 1.4.2.2 Interaction with endothelium

Acute inflammation is characterized by infiltration and accumulation of polymorphonuclear leukocytes (PMN) or neutrophils into the inflamed tissue (Soehnlein et al., 2009). The first wave of neutrophils dominates the inflamed tissue for the first three hours, which is followed by a second wave of emigrating monocytes (Rebuck and Crowley, 1955). In sub-acute and chronic inflammatory reactions monocytes and lymphocytes are the dominating extravasated cells. Azurocidin is stored in both azurophilic/primary granules and secretory vesicles of neutrophils and can be almost completely released into the extracellular environment following neutrophil activation (Tapper et al., 2002). The secretory vesicles are rapidly mobilized upon interaction of neutrophils with the vessel wall and azurocidin is the only granule protein released from these vesicles (Soehnlein et al., 2008; Tapper et al., 2002). Azurocidin is a potent chemoattractant for monocytes (Pereira et al., 1990b) and the rapid discharge



of azurocidin is linked to the early recruitment of inflammatory monocytes (Soehnlein et al., 2008). Azurocidin is also stored together with elastase, cathepsin G and proteinase-3 in the azurophilic/primary granules which are released slowly and with low tendency into the extravascular space (Tapper et al., 2002).

Adhesion of neutrophils to the endothelium causes release of azurocidin, which is mediated by leukocytic  $\beta 2$  integrin signalling (Gautam et al., 2001). The molecule of azurocidin has a strong dipole moment caused by basic amino acids concentrated on one side of the molecule (Iversen et al., 1997). The basic patch of secreted azurocidin binds to negatively charged proteoglycans on the endothelial cell membrane (Olofsson et al., 1999), which induces  $\text{Ca}^{2+}$ -dependent cytoskeletal rearrangement, cell contraction and increased permeability of the endothelium (Gautam et al., 2001). Azurocidin deposited on endothelium enhances monocyte arrest (Soehnlein et al., 2005) and contributes to the up-regulation of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) on endothelial cells, which promotes interactions of the endothelium with neutrophils and monocytes (Lee et al., 2003). Endothelium-bound azurocidin is internalized and protects endothelial cells from apoptosis (Olofsson et al., 1999). The exact mechanisms of azurocidin signalling is not determined (Linder et al., 2010), however, azurocidin has been shown to activate protein kinase C in endothelial cells (Pereira et al., 1996).

Binding of azurocidin to the protease inhibitor aprotinin inhibits permeability changes induced by azurocidin (Gautam et al., 2001; Petersen et al., 1993).

#### *1.4.2.3 Chemotactic activity*

Azurocidin was first shown to have chemotactic effects on monocytes and fibroblasts (Flodgaard et al., 1991b). It is also a chemoattractant of T lymphocytes, neutrophils and microglia, the mononuclear phagocytic cell of the central nervous system (Chertov et al., 1996; Chertov et al., 1997; Pereira et al., 2003). Secreted azurocidin can bind to monocytes, which results in internalization of azurocidin (Heinzelmann et al., 1999; Heinzelmann et al., 1998). This has shown to prolong monocyte survival and to potentiate LPS-induced production of proinflammatory cytokines e.g. tumor necrosis factor-alpha (TNF- $\alpha$ ) and IL-6 production from monocytes (Heinzelmann et al., 1999; Ostergaard and Flodgaard, 1992).

#### *1.4.2.4 Expression in chronic inflammation and in non-PMN cells*

Azurocidin is an important neutrophil-derived inflammatory mediator in acute inflammation. In addition, azurocidin has been detected in chronic inflammation and can be induced by proinflammatory cytokines and LPS in other types of cells. Azurocidin has been identified in endothelial cells (Lee et al., 2002) and smooth muscle cells (SMC) (Gonzalez et al., 2004; Pereira et al., 1996) of atherosclerotic plaques, and in endothelial cells in cerebral microvasculature in Alzheimer's disease. Expression of azurocidin in normal endothelium and SMC was not detected, but was induced in endothelial cells in response to TNF- $\alpha$ , IL-1 $\alpha$  and LPS (Lee et al., 2002; Pereira et al., 1996) and in smooth muscle cells in response to platelet-derived growth factor (PDGF) (Gonzalez et al., 2004).

The expression of azurocidin in SMC in atherosclerotic plaques was mainly observed in proliferating cells. Besides, azurocidin was found to stimulate proliferation and migration in vitro in aorta smooth muscle cells and to increase the levels of ICAM-1 (Gonzalez et al., 2004).

Pereira et al. studied the effect of azurocidin on corneal wound healing and found that azurocidin stimulated proliferation, migration and that adhesion molecules (ICAM-1, PECAM-1) and integrin molecules ( $\alpha$ 3,  $\beta$ 1) were up-regulated in human corneal epithelial cells (Pereira et al., 2004). The expression of azurocidin was inducible in corneal epithelium by TNF- $\alpha$  and IL-1 $\beta$  (Ruan et al., 2002). This indicates that azurocidin is involved in diseases involving inflammation.

A recent study has shown that monocytes stimulated with LPS can secrete azurocidin (Schou et al., 2011).

## **2 AIMS**

1. To further characterize a specific IGFBP-1 protease activity purified from a patient's urine, and to explore whether IGFBP-1 protease activity and IGFBP-1 fragments were presents in serum of the patient.
2. To determine expression of azurocidin and components of the IGF-system in human mast cells.
3. To determine any proteolytic effects of neutrophil-derived azurocidin on the six different IGFBPs, focusing especially on IGFBP-1.
4. To determine glycosylation and size of proteolytically active neutrophil-derived azurocidin.
5. To study biological functions of the IGFBP-1 and IGFBP-2 fragments on cell migration and on IGF-stimulated cell proliferation of primary human dermal fibroblasts.

## 3 METHODOLOGY

### 3.1 MATERIALS

All peptides used were the human isoforms. Recombinant full-length non-phosphorylated IGFBP-1 and phosphorylated IGFBP-1 were gifts from Kabi Pharmacia (Sweden). Highly phosphorylated HepG2-purified IGFBP-1 and a dephosphorylated preparation from the same source were purchased from Sigma–Aldrich (Sweden). Recombinant npIGFBP-1 and pIGFBP-1 were iodinated by the chloramine T method and purified on a PD-10 G25 column (Amersham Biosciences, UK). Recombinant pIGFBP-1 was biotinylated at the N-terminus with sulfo-NHS-LC-LC-biotin (EZ-Link, Pierce, IL, USA) according to the product instructions.

Human neutrophil-derived azurocidin and a rabbit polyclonal antibody against human neutrophil-derived azurocidin were purchased from Athens Research and Technology (Athens, Georgia, USA). Recombinant IGF-I (used in *paper I*) was a gift from Kabi Pharmacia (Sweden). IGF-II was donated by Eli Lilly (Indianapolis, IN). The recombinant proteins; glycosylated IGFBP-3, glycosylated IGFBP-4, glycosylated IGFBP-5, IGFBP-6, IGF-I (used in *paper III*), biotinylated IGF-I and biotinylated IGF-II and native IGFBP-1 purified from amniotic fluid, were from GroPep Limited (Adelaide, Australia). Recombinant IGFBP-2 was from Sandoz (Basel, Switzerland) and kindly provided by Christine Carlsson-Skwirut.

*In paper I*, two polyclonal rabbit anti-human IGFBP-1 antibodies were used, both of which had been raised against pIGFBP-1. One, KH103, was raised in-house (Povoa et al., 1984b) and the other, SU12, by Medprobe (Norway). Monoclonal antibodies to human IGFBP-1, mab6303 and mab6305, were gifts from Riitta Koistinen (Helsinki University Central Hospital, Finland) and were provided in a concentration of 1 µg/ml. *In paper III*, the mouse monoclonal anti-human integrin  $\alpha 5\beta 1$  antibody was from Abcam (Cambridge, UK).

### 3.2 CLINICAL SAMPLES

Urine and serum samples were obtained from a patient, a 73-year old woman with multiple myeloma and a long history of atopy with inflammatory skin lesions and eosinophilia (Wang et al., 2006b). Serum creatinine and cystatin C concentrations were

within the normal range for her age. Control serum was pooled from 6 healthy postmenopausal women. The study was approved by the local Ethics Committee.

### **3.3 PURIFICATION OF PROTEASE ACTIVITY FROM URINE**

The IGFBP-1 protease was purified by heparin-affinity chromatography as previously described (Wang et al., 2006b). In brief, urine from the patient was diluted 1:5 in 10 mM sodium phosphate, with 0.25 M NaCl, pH 7, and incubated in batches with heparin-agarose gel (Amersham Biosciences) for 30 min with continuous stirring. After washing the gel with 10 volumes of loading buffer, the salt content was increased to 2 M NaCl. The eluate was desalted (to 0.2 M NaCl), concentrated using a 10 kDa cut-off Microsep™ spin column (PALL Life Sciences, USA), and stored at - 80°C.

### **3.4 PROTEIN ANALYSIS**

Protein identification and N-Glycosylation site analysis of the neutrophil-derived azurocidin preparation was performed by MALDI-TOF peptide mass fingerprint and MALDI-TOF/TOF peptide sequencing by Alphalyse A/S (Odense, Denmark). To determine N-linked glycosylation sites a sample of azurocidin was incubated with PNGase F to remove N-glycans before sequencing and compared to non-treated azurocidin. The preparation was also analysed by Hans Jörnvall's laboratory using HPLC-separation, followed by Edman degradation.

### **3.5 PROTEOLYSIS OF PROTEINS**

Proteolysis of IGFBPs was carried out by incubation with azurocidin in phosphate-buffered saline (PBS) pH 7.0 for 2 h at 37 °C in a final volume of 10 µL. In *Paper II*, incubation of IGFBP-1 bound to IGF-I or IGF-II was performed in the same manner after cross-linking, using disuccinimidyl suberate (DSS) (Pierce, Rockford, IL) according to the manufacturer's instructions. The effect of IGFs on the proteolysis of IGFBP-1 was determined by incubating biotinylated IGFBP-1 with IGF-I or IGF-II for 2 h before adding azurocidin. These samples were not cross-linked. Thirteen nM of biotinylated IGF-I or IGF-II was incubated with increasing concentrations of azurocidin, elastase, cathepsin G or proteinase 3 in PBS in a final volume of 10 µl for 2 h at 37 °C.

The effect of IGFBP-1 fragments and IGFBP-2 fragments on migration and proliferation were studied in *paper III*. These fragments were obtained by incubating IGFBP (20 ng/μl) with azurocidin at a 1:1 molar ratio of IGFBP to azurocidin in PBS pH 7.0 for 2 hours at 37° C in a final volume of 90 μl.

### 3.6 SDS-PAGE

Samples were separated on SDS-PAGE under non-reduced or reduced conditions after proteolysis. Biotinylated proteins were visualized using neutravidin-horseradish peroxidase (Pierce) and detected by ECL (Amersham Biosciences, Uppsala, Sweden). S<sup>35</sup>-labeled proteins were visualized by autoradiography. Non-biotinylated IGFBPs and azurocidin were separated on SDS-PAGE and detected by silver staining using the SilverQuest kit (Invitrogen, Stockholm, Sweden). Densitometric analysis of stained bands was conducted using the software NIH ImageJ (<http://rsb.info.nih.gov/ij/>).

### 3.7 IN VITRO TRANSCRIPTION/TRANSLATION OF IGFBP-1 FRAGMENTS

In vitro-translated proteins corresponding to the IGFBP-1 fragments generated by specific protease activity were produced in order to study their size and immunoreactivity, compared to intact IGFBP-1. Total RNA isolated from HepG2 cells was reverse-transcribed and first-strand cDNAs were used for PCR. Oligonucleotide primers for PCR of N-terminal recIGFBP-1<sup>1-130</sup> fragment: forward primer 50-TTCTCGAGATGTCAGAGGTCCCCGTTGCT-30 and reverse primer 50-TTTCTAGAT TAGATGGCGTCCCAAAGGATGGA-30 and of C-terminal recIGFBP-1<sup>131-234</sup> fragment: forward primer 50-TTCTCGAGATGAGTACCTATGATGGCTCGAAG-30 and reverse primer 50-CCTCTAGACATCTGGTTTCAGTTTTGTACATTAAA-30. The forward primers contain a restriction site for XhoI and reverse primers a restriction site for XbaI. The PCR cycles for the N-terminal fragment were as follows: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 10 min. The PCR cycles for the C-terminal fragment were as follows: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 10 min. The N-terminal (483 bp) and the C-terminal fragment (334 bp) PCR products were gel-purified and TA cloned according to the manufacturer's instructions (Invitrogen, Sweden). Plasmid DNA was purified (E.Z.N.A Plasmid Mini Kit I,

Omega) and sequenced using Big Dye Terminator Kit and an automatic sequencer (both from Genetic Analyzer 3100, Applied Biosystems). Plasmids containing the correct inserts were subsequently cleaved with XhoI and XbaI and cloned into the pTNT vector (Promega) according to the product instructions. Plasmid DNA was purified, sequenced and used for in vitro transcription/translation using the TNT Quick Coupled Transcription/Translation Systems (Promega) with incorporation of 35S-labeled methionine. Samples of labeled products were separated by 12% SDS-PAGE, subjected to autoradiography and analyzed on a PhosphorImager scanner (Fuji film Bas-2500). The immunoreactivity of the in vitro translated products was analyzed by RIA as described below.

### **3.8 SIZE-SEPARATION COLUMN CHROMATOGRAPHY**

IGFBP-1 fragments were size-separated under non-denaturing conditions. Samples containing <sup>125</sup>I-pIGFBP-1 (180,000 cpm with 22 µl serum), in a final volume of 400 µl in column buffer (0.05 M PBS, pH 7) or cleaved biotinylated pIGFBP-1 in 400 µl column buffer were applied to a Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated with column buffer. The flow rate was 400 µl/min, and 1 min fractions were collected. The column was calibrated with standards from Amersham Biosciences (BSA, chymotrypsinogen and ribonuclease A) and recIGF-I (a gift from Kabi Pharmacia Sweden, to K.H.). Samples containing IGFBP-1 and its fragments were analyzed by gamma counting, chemiluminescence after SDS-PAGE or radioimmunoassay, as appropriate.

### **3.9 RADIOIMMUNOASSAYS**

Four radioimmunoassays were used; an in-house RIA using a polyclonal antibody raised against pIGFBP-1 (KH103), previously described (Povoa et al., 1984b), and three new RIAs, using the polyclonal antibody SU12 (1:36 000/1:18 000) and two monoclonal antibodies, 6303 (1:720 000) and 6305 (1:720 000). In each assay recombinant npIGFBP-1 was iodinated and used as tracer (8000 cpm/assay tube). Samples were incubated with antibody and tracer in Tris assay buffer, 1% bovine serum albumin, in a final volume of 300 µl. After overnight incubation at 4 °C, bound counts were separated with goat anti-rabbit, or goat anti-mouse antibody as appropriate (SacCel, Boldon, England).

### **3.10 LIGAND-BINDING ASSAYS**

Ligand-binding was performed by incubating biotinylated IGFBP-1 with an excess of IGF-I or IGF-II in PBS pH 7.0 at 22 °C for 2 h followed by cross-linking, using a final concentration of 0.25 mM DSS. The samples were divided equally into four Eppendorf tubes and proteolysis was carried out by incubating with 0, 1, 10 or 100 ng of azurocidin in a final volume of 10 µL.

### **3.11 CELL CULTURE**

Primary human dermal fibroblasts from two donors (HDF) (Promocell, Germany) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Sweden), 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin at 37°C in 5% CO<sub>2</sub> humidified environment. Cell studies were performed between passage numbers 4 and 7. Human mast cells (HMC-1) (kindly provided by J.H Butterfield at the Mayo Clinic) cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 1 .2 mM alpha thioglycerol and 10% FBS.

### **3.12 THYMIDINE INCORPORATION**

Proliferation of fibroblasts was determined by quantifying DNA synthesis through the incorporation of [3H] thymidine. Fibroblasts were plated in 24-well plates and experiments were carried out at approximately 30% or 60% confluence. Cells were incubated in serum-free medium for 24 h before adding the different media conditions for a further 22 h, with [3H] thymidine (1 µCi/ml) added for the last 4 h. The medium was removed and cells were rinsed twice with 1 mL ice-cold 0.09% NaCl and then incubated with 1 mL ice-cold 5% trichloroacetic acid (TCA) for 15 min. The fixed nucleotides were solubilized in 0.5 mL of 0.1 M NaOH for 2 h at room temperature under constant shaking. The radioactivity was measured in a β-counter after the addition of Ultima Gold scintillation liquid to the samples.

### **3.13 IN VITRO MIGRATION ASSAY**

Cell migration of fibroblasts was studied using the in vitro “scratch” assay earlier described by Li et al. 2004 (Li et al., 2004). Twelve-well plates were pre-coated with collagen (50 µg/ml) and blocked with 3% BSA. A sufficient number of cells were plated to reach confluence right after attachment of cells. Cells were starved over-night



and scratches were made the following day by using a micropipette tip. Floating cells were removed by washing with PBS before media containing 0.2% FBS and different experimental conditions were added. Mitomycin C (10  $\mu$ l/ml) was included in the media to prevent cell proliferation. Scratches were photographed with an inverted phase microscope before and after the 7 h treatment and migration was calculated from the cell-free area after treatment relative to before treatment, using software NIH ImageJ (<http://rsb.info.nih.gov/ij/>). To examine the involvement of the RGD-sequence on migration, cells were pre-incubated with a monoclonal  $\alpha$ 5 $\beta$ 1 integrin antibody (diluted 1:1000) 30 minutes prior to treatment.

### **3.14 STATISTIC ANALYSES**

Statistical analyses were performed using GraphPad Prism version 5.04, GraphPad Software (San Diego, CA, USA). The values are the mean  $\pm$  SEM. All statistical tests were performed with one-way ANOVA or unpaired Student *t*-test and followed by Bonferroni post-test or Dunnett's Multiple Comparison Test when appropriate. Results with a P-value of  $< 0.05$  were considered statistically significant. Significance is denoted as \* $<0.05$ , \*\* $<0.01$  and \*\*\* $<0.001$ .

## 4 RESULTS AND DISCUSSION

### 4.1 CHARACTERIZATION OF IGFBP-1 PROTEOLYSIS IN A PATIENT (PAPER I)

Our group has previously purified an IGFBP-1 specific protease activity from the urine of a 73 year old patient with multiple myeloma and an inflammatory skin disease (Wang et al., 2006b). This activity cleaved both phosphorylated and non-phosphorylated IGFBP-1 efficiently at a unique site (Ile<sup>130</sup>–Ser<sup>131</sup>). The protease activity was identified as azurocidin, based on the presence of azurocidin in partially purified material from urine and the inhibitory effect of an antibody to azurocidin. However, the protease activity of azurocidin was not isolated in its active form and it was destroyed by reverse phase HPLC (Wang et al., 2006b).

Since azurocidin previously has been reported to be proteolytically inactive (Campanelli et al., 1990b; Pereira et al., 1990b; Wilde et al., 1990b), the activity of the patient-derived azurocidin was speculated to be unique.

#### 4.1.1 Size-analysis of IGFBP-1 fragments

IGFBP-1 fragments generated by the partially purified material of azurocidin (ppm) were previously purified on HPLC and peaks were sequenced and visualized on SDS-PAGE (Wang et al., 2006b). Two main fragments were detected. The N-terminal fragment (IGFBP-1<sup>1-130</sup>) migrated at approximately 20 kDa which is higher than its predicted molecular mass. The predicted molecular mass of the N-terminal fragment (IGFBP-1<sup>1-130</sup>) was 13.4 kDa on MALDI-MS; while a C-terminal fragment (IGFBP-1<sup>142-234</sup>) had a mass of 10.8 kDa on MALDI-MS. The predicted molecular masses of the specific IGFBP-1 fragments, based on amino acid compositions, are 13.4 and 10.9 kDa, for IGFBP-1<sup>1-130</sup> and IGFBP-1<sup>142-234</sup> respectively. The observed masses (on SDS-PAGE and mass spectrometry) and theoretical masses of the N- and C-terminal IGFBP-1 fragments are shown in **Table 3**.

**Table 3.** Comparison of observed and theoretical masses of intact, N- and C-terminal fragments of IGFBP-1.

	SDS-PAGE	Mass spectrometry	Theoretical mass
<b>Intact pIGFBP-1</b>	28–30 kDa	25.2 kDa	25.3 kDa
<b>N-terminal fragment</b>			
<i>In vitro</i> (N-IGFBP-1 <sup>1–130</sup> ) <sup>a</sup>	21.5 kDa	N.D.	13.4 kDa
Purified (IGFBP-1 <sup>1–130</sup> )	20 kDa <sup>b</sup>	13.4 kDa	13.4 kDa
<b>C-terminal fragment</b>			
<i>In vitro</i> (C-IGFBP-1 <sup>131–234</sup> ) <sup>a</sup>	11.4 kDa	N.D.	11.9 kDa
Purified (IGFBP-1 <sup>142–234</sup> )	14 kDa <sup>b</sup>	10.8 kDa	10.9 kDa

Molecular masses of intact and cleaved IGFBP-1 were determined by SDS–PAGE and mass spectrometry. The theoretical masses were predicted from the amino acid composition. N.D., not determined.

<sup>a</sup> Translated *in vitro*.

<sup>b</sup> Fragments were purified by HPLC by Wang, Shafqat et al.

Biotinylated pIGFBP-1 was cleaved with the IGFBP-1 protease preparation and then size-separated by FPLC under neutral conditions. The presence of biotin (intact IGFBP-1 and any fragments containing the N-terminus) was then detected after SDS–PAGE. When analyzed by RIA, using antibody SU12, the elution profile was complex and appeared to represent several immunoreactive peaks over a broad range of molecular masses, with most immunoreactive fragments eluting around 13 kDa.

In order to study the discrepancy in the molecular size of the IGFBP-1 fragments, *in vitro* translated forms were produced and analyzed on SDS–PAGE. The N-terminal fragment (IGFBP-1<sup>1–130</sup>) migrated at an apparent molecular mass of 21.5 kDa under reducing conditions, which is a similar molecular mass to that generated by cleavage of N-terminally biotinylated IGFBP-1 (Wang et al., 2006b). The C-terminal IGFBP-1<sup>131–234</sup> had an observed molecular mass of 11.4 kDa, close to its theoretical mass of 11.9 kDa. IGFBP fragments may run anomalously on SDS–PAGE. An N-terminal fragment of IGFBP-3 has been observed to migrate at a molecular mass higher than its actual size (Salahifar et al., 2000).

#### 4.1.2 Immunoreactivity of IGFBP-1 fragments

Specific cleavage had little effect on total IGFBP-1 immunoreactivity in three of the RIAs. However in one RIA, using monoclonal antibody mab6305, there was a clear loss of immunoreactivity. Surprisingly in this assay the recombinant C-terminal fragment effectively displaced IGFBP-1 tracer. Since the C-terminal fragment can

undergo further proteolysis in the presence of azurocidin (Wang et al., 2006b), this may explain the loss of immunoreactivity in an assay that is directed to the C-terminus. Indeed C-terminal fragments of approximately 7 kDa were evident after cleavage of radiolabeled IGFBP-1.

#### **4.1.3 Detection of proteolytic activity and fragments in serum**

We found that the IGFBP-1-specific protease activity was completely abolished in the presence of serum from the patient. This is not surprising since a number of protease inhibitors are present in the circulation. It has been reported that alpha2-macroglobulin binds to IGFBP-1 and inhibits the effect of MMP-3 on IGFBP-1 cleavage (Westwood et al., 2001). Wang et al. have previously shown that this IGFBP-1-specific protease activity was inhibited by serine protease inhibitors (Wang et al., 2006b). IGFBP-3 proteases have been reported to be inhibited by serum inhibitors (Maile et al., 2000) and it has been proposed that specific degradation of IGFBP-3 (Koistinen et al., 2002) and IGFBP-4 (Bayes-Genis et al., 2001) occur at the tissue level to increase local IGF action. Azurocidin is mobilized from neutrophil secretory granules in response to inflammation (Watorek, 2003). It is also expressed in activated endothelial cells associated with atherosclerotic plaques (Lee et al., 2002) and with Alzheimer's lesions (Pereira et al., 1996). Co-localization of azurocidin and IGFBP-1 in these or other sites may regulate IGFBP-1 activity locally. Since no IGFBP-1 protease activity was detected in the patient serum, activity of this protease is therefore likely to have a tissue-specific role. The protease could reach the tissues in an endocrine fashion, or may be produced locally; and IGFBP-1 fragments generated in tissues may enter the circulation.

The IGFBP-1-specific protease activity was purified from urine; therefore kidney could be a potential source. However, the patient was diagnosed with two inflammatory diseases, multiple myeloma and dermatitis, and the activity was co-purified with two other neutrophil proteins (Wang et al., 2006b), which suggests that it was derived from neutrophils.

Although there was no circulating protease activity in serum we have evidence that immunoreactive fragments of IGFBP-1 are present in the circulation of our patient. Circulating IGFBP fragments have previously been reported, however, their presence is usually associated with renal failure (Kiepe et al., 2001) and our patient had normal renal function.

*In summary*, specific cleavage of IGFBP-1 probably occurs at the tissue level and not in the circulation in a patient with multiple myeloma and dermatitis. The fragments that are generated may have endocrine roles.

## **4.2 PROTEOLYTIC EFFECTS OF NEUTROPHIL-DERIVED AZUROCIDIN (PAPER II AND III)**

### **4.2.1 Neutrophil-derived azurocidin cleaves IGFBP-1,-2 and -4 (paper II)**

We found that a commercially available preparation of neutrophil-derived azurocidin cleaved recombinant phosphorylated IGFBP-1 into three fragments of 22, 20 and 14 kDa. The fragments were of equal sizes as obtained when phosphorylated IGFBP-1 was cleaved with urine-derived (from patient) azurocidin (Wang et al., 2006b). See section 4.5.1 for a comparison between urine-derived and neutrophil-derived azurocidin.

The effect of neutrophil-derived azurocidin on native IGFBP-1 purified from amniotic fluid was also studied and detected by silver staining. Native IGFBP-1, which consists of three to four phosphorylated forms of IGFBP-1, was cleaved into three fragments of 23, 20 and 14 kDa. The proteolytic effect was detected at picomolar concentrations of azurocidin which is considerably less than required for other effects of azurocidin. Antimicrobial and chemotactic effects are exerted by azurocidin at  $\mu\text{M}$  concentrations. The proteolytic effect of neutrophil-derived azurocidin was inhibited by a polyclonal antibody against azurocidin (see section 4.5.1).

Neutrophil-derived azurocidin also cleaved IGFBP-2 and IGFBP-4. Incubation of IGFBP-2 with azurocidin resulted in two fragments of approximately 23 and 19 kDa, while glycosylated IGFBP-4 of 24–28 kDa was cleaved into one major fragment of approximately 17 kDa and one minor fragment of approximately 15 kDa. Azurocidin was previously reported to be an IGFBP-1 specific protease (Wang et al., 2006b). The contradictory results could be due to different methods of detection. In the former report radioiodinated IGF-I was used to visualize IGFBPs and possible fragments, while we used silver staining. It has been shown that IGFBP-4 fragments produced by the protease PAPP-A have markedly reduced affinity for IGFs compared to intact IGFBP-4 (Boldt and Conover, 2007). Proteolytic fragments of IGFBP-2 have also been shown to have decreased affinity for IGFs compared with intact protein (Firth and Baxter, 2002a). In **paper III**, we show that proteolysis of phosphorylated IGFBP-1 did not

alter its inhibitory effects on IGF-induced proliferation, which could be due to efficient binding between IGFBP-1 fragments and IGFs.

#### **4.2.2 Effects of IGFs on IGFBP-1 proteolysis (paper II)**

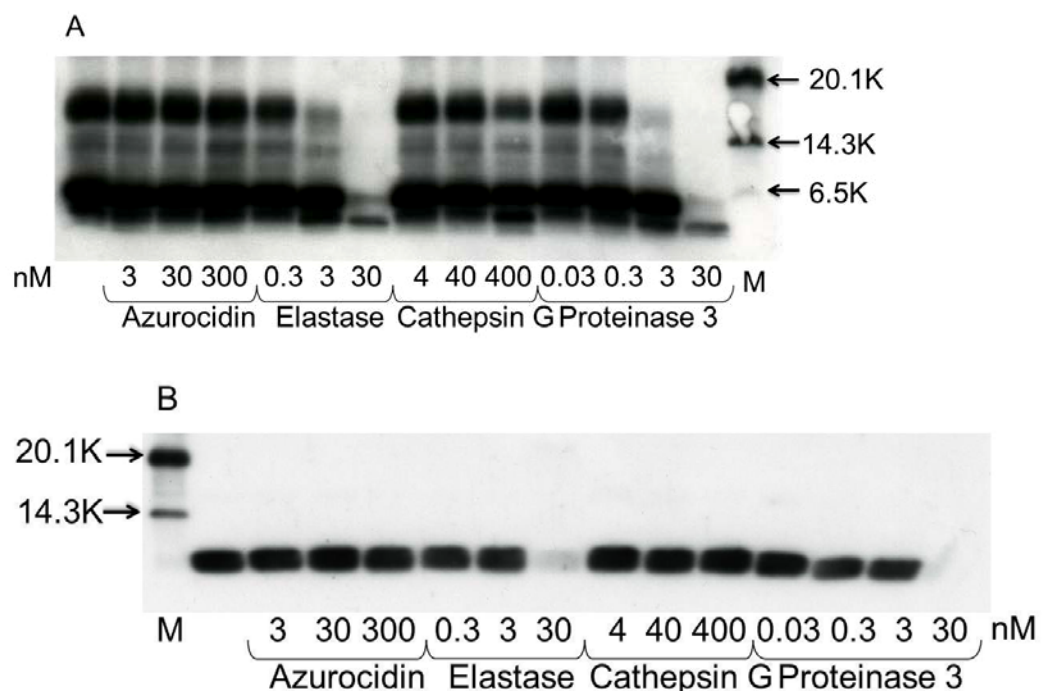
Phosphorylated IGFBP-1 has higher affinity for IGFs compared to less phosphorylated IGFBP-1 and has been reported to be resistant to proteolysis (Gibson et al., 2001). To determine the effect of the neutrophil-derived azurocidin preparation on IGF-bound phosphorylated IGFBP-1, IGFBP-1 was ligand-bound and cross-linked to IGF-I or IGF-II before being incubated with the azurocidin-preparation. We observed cleavage of phosphorylated IGFBP-1 when bound to IGF-I. Increasing concentrations of azurocidin reduced IGF-I / IGFBP-1 complexes and intact IGFBP-1, while the 22-kDa fragments increased. The amount of cleavage of IGF-I-bound IGFBP-1 was not possible to determine since both unbound and ligand-bound IGFBP-1 coexisted in the reaction tube and fragments might descend from both forms of IGFBP-1. However, the highest concentration of azurocidin resulted in almost complete loss of ligand-bound IGFBP-1, which supports proteolysis of IGF-I-bound IGFBP-1. It is uncertain if IGF-II-bound IGFBP-1 was cleaved or not. This complex appears faint compared to IGF-I-bound IGFBP-1, which might be due to less efficient ligand-binding. However, in the presence of IGF-II and in comparison to IGF-I, proteolysis of IGFBP-1 was significantly reduced. This method is not reliable to quantify and compare proteolysis of unbound and IGF-bound IGFBP-1 as the presence of DSS and an excess of IGFs could also have an impact on the proteolysis of IGFBP-1. The biotin molecule attached to IGFBP-1 could also be less visible on the blot when bound to IGFs due to conformational changes.

Since proteolysis of IGFBP-1 was reduced in reactions containing IGF-II, we studied the effect of IGF-I and IGF-II on IGFBP-1 proteolysis. The effect of IGFs on the proteolysis of IGFBP-1 was determined by pre-incubating IGF-I or IGF-II with IGFBP-1 for 2 h, but without cross-linking proteins, before incubating with azurocidin. IGF-II showed inhibitory effect on IGFBP-1 proteolysis, while IGF-I had no effect on cleavage of IGFBP-1. Proteolysis in the absence of IGFs resulted in 80% cleavage of intact IGFBP-1. At a 1:1 ratio of IGFBP-1 to IGF-II, cleavage of intact IGFBP-1 was reduced by approximately 50% compared to proteolysis of intact IGFBP-1 in the absence of IGF-II.

### 4.2.3 Effects of neutrophil-derived azurocidin on IGFs (paper III)

The proteolytic activity of neutrophil-derived azurocidin towards IGF-I and IGF-II was examined to exclude degradation of these proteins, using the same concentrations of azurocidin which efficiently cleaved IGFBP-1, -2 and -4 in **paper II**. We have also compared the effect of azurocidin to three closely structurally related neutrophil proteases; elastase, proteinase 3 and cathepsin G (**Figure 2**). Elastase and Cathepsin G have earlier been reported to efficiently cleave all six IGFBPs and have been speculated to be involved in the regulation of IGFs and IGFBPs during inflammation and wound healing (Gibson and Cohen, 1999). Azurocidin had no proteolytic effect on IGF- I or IGF-II with the used concentrations. In contrast to azurocidin, we observed cleavage of IGF-I and IGF-II by elastase and proteinase 3. Cathepsin G showed proteolytic activity towards IGF-I, but not IGF-II.

*In summary*, the neutrophil-derived preparation of azurocidin cleaved IGFBP-1, IGFBP-2 and IGFBP-4. Phosphorylated IGFBP-1 bound to IGF-I was also degraded whereas IGF-II was shown to have an inhibitory effect on proteolysis of phosphorylated IGFBP-1. The proteolytically active preparation of neutrophil-derived azurocidin was found to be glycosylated and determined to be 31 kDa by SDS-PAGE.



**Figure 2.** Proteolysis of biotinylated IGFs by azurocidin, elastase, cathepsin G or proteinase 3. Thirteen nM (1 ng) IGF-I (A) or IGF-II (B) were incubated with the indicated concentration of protease for 2 h at 37°C. Proteins were separated using SDS-PAGE and blotted onto nitrocellulose membrane. Biotinylated IGFs were visualized by neutravidin-horseradish peroxidase and detected by ECL. The apparent molecular weights of biotinylated protein standard (M) are indicated.

### 4.3 ANALYSIS OF NEUTROPHIL-DERIVED AZUROCIDIN

This commercially available preparation of azurocidin was purified from neutrophils by affinity chromatography and activity assays had been performed for proteinase 3, elastase and cathepsin G by Athen's Research and Technology to rule out contamination of these. In **paper II** we confirmed the identity of the preparation by protein sequencing and performed N-Glycosylation site analysis by MALDI-TOF peptide mass finger print and MALDI-TOF/TOF peptide sequencing.

The preparation of neutrophil-derived azurocidin was further analysed by HPLC and Edman degradation (not included in papers). Intact azurocidin was found to be the main component (approximately 75%). Two fragments of azurocidin were also detected in the preparation, one starting from amino acid 41 and the other from amino acid 106, which could be the result of autocatalytic activity. A fourth component was found and has not been described in the literature earlier.

Elastase was not detected with these methods and could therefore only be present in low concentrations. In addition, elastase (0.3 nM) cleaved both IGF-I and IGF-II, while the highest concentration of the azurocidin-preparation (300 nM) showed no effects on IGF-I and IGF-II, therefore any contamination of elastase must be less than 0.1% of the preparation of azurocidin.

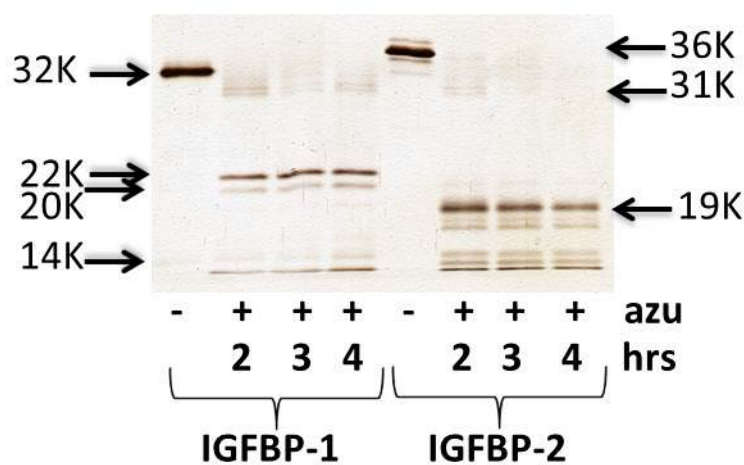
Azurocidin contains three N-linked glycosylation sites that are located at asparagine residues 100, 114 and 145. Different molecular masses have been reported (29–37 kDa), which is probably due to different degrees of glycosylation (Watorek, 2003). Neutrophil-derived azurocidin was glycosylated at asparagine 145 and determined to be 31 kDa by SDS-PAGE (**paper II**).

### 4.4 BIOLOGICAL EFFECTS OF IGFBP-1 AND IGFBP-2 FRAGMENTS (PAPER III)

The IGF-system is important in wound healing (Skottner et al., 1988) and our results indicate that the inflammatory mediator azurocidin can cleave IGFBP-1, IGFBP-2 and IGFBP-4. The C-terminal of IGFBP-1 and IGFBP-2 contain an Arg-Gly-Asp (RGD) sequence which can bind to cell-surface  $\alpha 5 \beta 1$ -integrins (Brewer et al., 1988; Jones et al., 1993c). IGFBP-1 has been shown to increase migration independently of IGF-I by activating  $\alpha 5 \beta 1$ -integrins (Gleeson et al., 2001; Jones et al., 1993c; Perks et al., 1999), while there are only a few reports of such IGFBP-2 effects (Wheatcroft and Kearney, 2009). In contrast to IGFBP-4, IGFBP-1 and IGFBP-2 are



cleaved into stable fragments by the neutrophil-derived preparation of azurocidin and contain a RGD-sequence (*paper II*). We therefore studied the effect of IGFBP-1, IGFBP-2 and their fragments on migration and proliferation, which are important in wound healing. Fibroblasts were used as they are involved in the inflammatory process and maintain the extracellular matrix (Glaros et al., 2009). To obtain fragments for these studies, IGFBP-1 and IGFBP-2 were incubated with the neutrophil-derived preparation of azurocidin until (2 h) all intact protein was degraded into fragments (**Figure 3**).



**Figure 3.** Proteolysis of native IGFBP-1 and recombinant IGFBP-2. After the times indicated, proteins were detected by silver staining after SDS-PAGE. Azurocidin appears at 31 kDa.

#### 4.4.1 Effects on migration of fibroblasts

We studied the effect of IGFBP-1, IGFBP-2 and their fragments on migration of human dermal fibroblasts for 7h by using the in vitro “scratch” assay earlier described by Li et al. 2004 (Li et al., 2004). IGFBP-1 fragments and IGFBP-2 fragments stimulated migration of fibroblasts as efficiently as intact IGFBP-1 and IGFBP-2. IGFBP-1 and IGFBP-1 fragments stimulated migration of fibroblasts by around 40%, while IGFBP-2 and its fragments increased migration by approximately 50% compared to controls. The effect of azurocidin was also determined as the preparations of fragments contained azurocidin, but no effect was detected. These results support another study by Sala et al., which demonstrated that a C-terminal fragment isolated

from amniotic fluid retains the capability of intact IGFBP-1 to stimulate cell migration (Sala et al., 2005).

To determine whether the increased migration involved binding of IGFBP C-terminal RGD-sequence to  $\alpha 5 \beta 1$  integrin, cells were pre-incubated with a monoclonal  $\alpha 5 \beta 1$  integrin antibody prior to the treatment with intact IGFBPs or fragments. The antibody abolished the stimulatory effect of IGFBP-1, IGFBP-2 and fragments on migration, which suggests that the increased migration involves an interaction of the RGD-sequence with  $\alpha 5 \beta 1$  integrins. IGFBP-1 stimulated migration has earlier been shown to be blocked by pre-incubating with an anti- $\alpha 5 \beta 1$  antibody (Gleeson et al., 2001).

#### **4.4.2 High glucose decrease migration of fibroblasts**

High glucose (25 mM) decreased migration of fibroblasts by approximately 45% compared to migration in normal glucose. This supports an earlier study where high glucose levels (25 mM) reduced migration of cardiac fibroblasts (Zhang et al., 2007). The addition of IGFBP-1, IGFBP-2, IGFBP-1 fragments or IGFBP-2 fragments increased migration in high glucose to normal levels, equivalent to migration in normal glucose.

#### **4.4.3 Effects on IGF-I stimulated proliferation**

The effect of IGFBP-1 on IGF-I stimulated proliferation of human dermal fibroblasts appeared complex. We have observed two different outcomes depending on how confluent the cells were at the time of treatment. IGFBP-1 had no inhibitory effect on IGF-I stimulation when cells were sparsely plated and approximately 30% at the time of experiment. Instead IGFBP-1 potentiated IGF-I activity. IGFBP-1 had no potentiating effects on IGF-II (not shown), which suggests that this might be a selective effect of IGF-I. These results are supported by two other studies where IGFBP-1 potentiated IGF-I stimulated proliferation of fetal and adult human fibroblasts, respectively (Koistinen et al., 1990; Kratz et al., 1992).

In experiments when cells were sparsely plated, we also observed increased proliferation by the addition of IGFBP-1 or azurocidin alone. Azurocidin and IGFBP-1 stimulate proliferation more than 2-fold compared to controls. Azurocidin-increased proliferation could be due to cleavage of endogenously produced IGFBPs and a subsequent increase in bioavailable IGFs or perhaps azurocidin stimulated proliferation on its own. Azurocidin has previously been reported to promote proliferation and to up-

regulate adhesion molecules (ICAM-1, PECAM-1) and integrin molecules ( $\alpha 3$ ,  $\beta 1$ ) in corneal epithelial cells (Pereira et al., 2004). Azurocidin was also found to stimulate proliferation and migration and to increase the levels of ICAM-1 in vitro in aorta smooth muscle cells (Gonzalez et al., 2004). The exact mechanisms azurocidin signalling is not determined (Linder et al., 2010). However, the molecule of azurocidin has a strong dipole moment caused by basic amino acids concentrated on one side of the molecule and has been shown to bind to (Iversen et al., 1997) negatively charged proteoglycans on the endothelial cell membrane (Olofsson et al., 1999). The stimulatory effect of IGFBP-1 could be due to potentiation of endogenously produced IGF-I or be the result of IGF-independent stimulation by IGFBP-1.

When cells were more confluent (approximately 60%) at the time of experiment, IGFBP-1 inhibited IGF-I stimulated proliferation, and at even higher densities the stimulatory effect of IGFs on fibroblasts disappeared (not shown). In these experiments neither IGFBP-1 nor azurocidin showed any stimulatory effects on proliferation of fibroblasts on their own

Proteolysis of phosphorylated IGFBP-1 had no effect on potentiating or inhibitory effects of IGFBP-1 on IGF-I stimulated proliferation since we observed similar results with the proteolytic fragments of IGFBP-1.

The phosphorylation state of IGFBP-1 has been reported to determine whether IGFBP-1 inhibits or potentiates IGF activity: phosphorylated IGFBP-1 increased binding affinity and favoured inhibition, while non-phosphorylated IGFBP-1 was associated with potentiation of IGF-I activity (Jones et al., 1991). According to our results, the state of phosphorylation of IGFBP-1 does not determine if IGFBP-1 inhibits or potentiates IGF action since we used phosphorylated IGFBP-1 and obtained both inhibitory and potentiating effects. Instead, we observed that the effects of IGFBP-1 on IGF activity was depending on cell confluence.

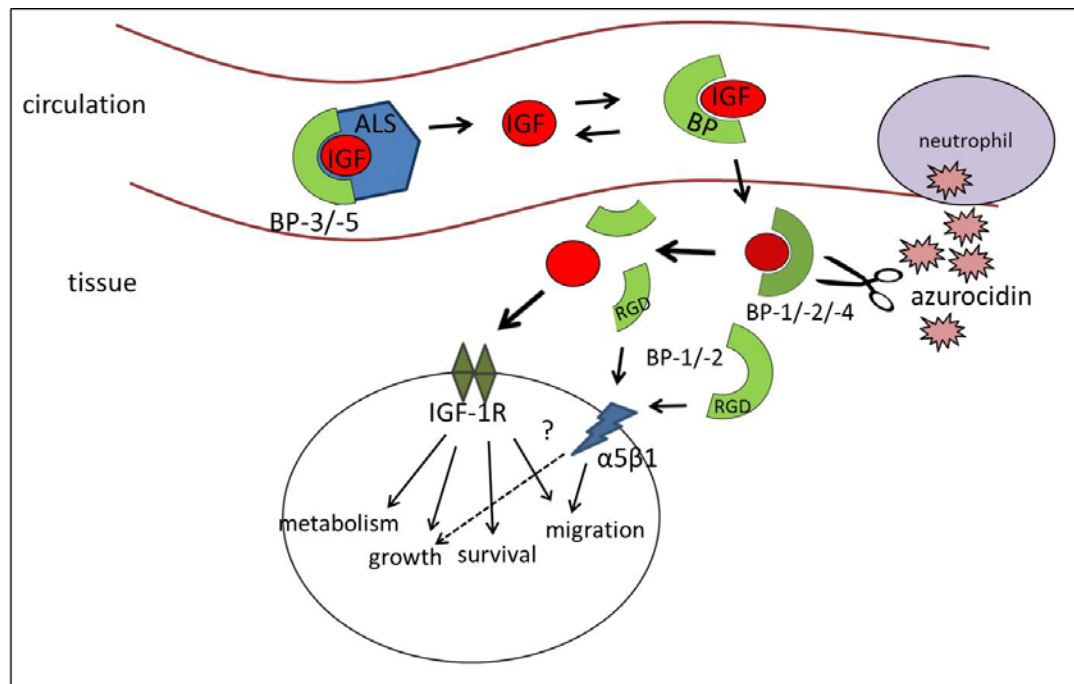
Human dermal fibroblasts have been reported to secrete IGF-I and IGFBP-3, but not IGFBP-1 (Conover et al., 1989). However, in this reported study binding-proteins were detected in conditioned medium from confluent cells. Another study has shown that human fetal fibroblasts release IGFBP-1, but only when sparsely plated and rapidly growing (Hill et al., 1989). Martin and Baxter have shown that secretion of IGFBP-1 from human neonatal fibroblasts disappeared as concentrations of exogenous IGFBP-3 increased (Martin and Baxter, 1992). It is therefore uncertain whether human dermal fibroblasts secrete IGFBP-1 or not when sparsely plated. The secretion of IGFs and IGFBPs from fibroblasts at different confluence and the interactions of these needs to

be further studied in order to understand the complexity of the IGF-system in wound healing.

#### **4.4.4 Effects on IGF-II stimulated proliferation**

The general function of IGFBP-2 is to inhibit IGF actions, in particular IGF-II, since it has higher affinity for IGF-II (Firth and Baxter, 2002b). Our results demonstrate that in contrast to IGFBP-1, proteolysis of IGFBP-2 affected the inhibitory effect on IGFs. Intact IGFBP-2 inhibited IGF-II induced proliferation while IGFBP-2 fragments had no significant inhibitory effects. It is possible that the N- and C-terminals of IGFBP-1 bind independently of each other to IGFs, while both ends of IGFBP-2 might be required for the same task. The N- and C-terminal domains of IGFBP-2 cooperate in IGF binding (Kuang et al., 2007).

*In summary*, IGFBP-1 fragments and IGFBP-2 fragments stimulated migration as efficiently as intact IGFBP-1 and IGFBP-2. The stimulatory effect was abolished by pre-treating cells with a  $\alpha 5\beta 1$  integrin antibody. High glucose impaired migration, however, the addition of IGFBP-1, IGFBP-2 or fragments increased migration to levels observed in normal glycaemia. IGFBP-2 inhibited IGF-II induced proliferation, whereas IGFBP-2 fragments had no significant inhibitory effect. The effect of intact IGFBP-1 and IGFBP-1 fragments on IGF-I induced proliferation had both potentiating and inhibitory effects, depending on the confluence of cells. We also observed stimulatory effects of both IGFBP-1 and azurocidin alone on proliferation of fibroblasts. The IGFBP-fragments obtained by incubating intact proteins with a neutrophil-derived preparation of azurocidin had effects on both migration and proliferation of human dermal fibroblasts, implicating effects of azurocidin and IGFBP-fragments on wound healing (**Figure 4**).



**Figure 4.** Proposed model for regulation of IGF-system in inflamed tissue. Adhesion of neutrophils to the endothelium causes release of azurocidin, which cleaves IGFBP-1, -2 and -4. Intact IGFBP-1 and -2 and their fragments stimulate migration.

## 4.5 RESULTS NOT INCLUDED IN PAPER I-III

### 4.5.1 Analysis of neutrophil-derived azurocidin

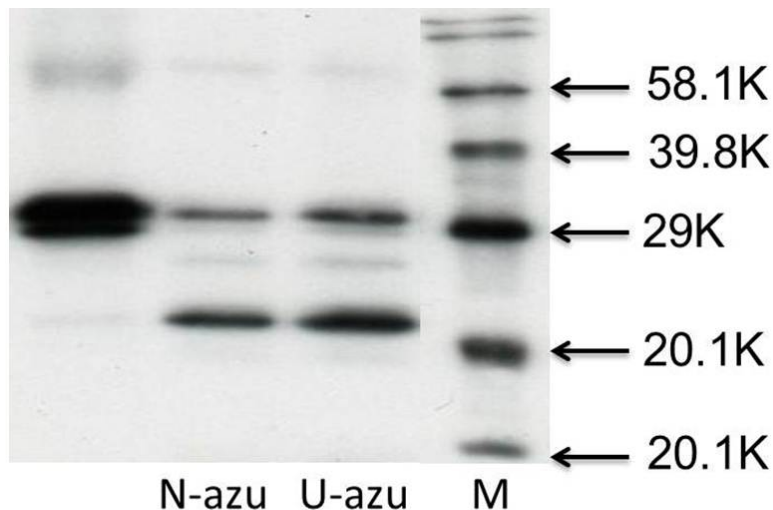
Azurocidin purified from the patient was earlier speculated to be unique as azurocidin previously has been reported to be proteolytically inactive. In **Figure 5**, biotinylated recombinant pIGFBP-1 was cleaved with either 10 ng of neutrophil-derived azurocidin or 1  $\mu$ l of partially purified azurocidin from the urine of our patient. Neutrophil-derived azurocidin cleaved approximately 84% of intact IGFBP-1 while urine-derived azurocidin degraded around 73%.

The proteolytic effect of neutrophil-derived azurocidin was blocked by a polyclonal antibody against azurocidin (**Figure 6**). Proteolysis of IGFBP-1 by neutrophil-derived elastase was also inhibited by this antibody, but only with the highest concentration of antibody and not in a dose-dependent manner as azurocidin.

Azurocidin can bind to LPS and neutralize its endotoxic effects (Brackett et al., 1997; Pereira et al., 1993). We studied the proteolytic effect of azurocidin when co-incubated with LPS and found no effect of LPS on proteolysis of IGFBP-1 by neutrophil-derived azurocidin



**Figure 6. Azurocidin antibody inhibits proteolysis of IGFBP-1.** Biotinylated pIGFBP-1 (1 ng) was incubated with 10 ng of N-azu at pH 7.0, 37 °C for 2 h with increasing concentrations of a polyclonal antibody towards azurocidin in a final volume of 10  $\mu$ L. Proteins were separated using SDS-PAGE and detected according to Materials and Methods.



**Figure 5. Proteolysis of phosphorylated IGFBP-1 by neutrophil-derived (N-azu) or urine-derived azurocidin (U-azu).** Biotinylated pIGFBP-1 (1 ng) was incubated with 10 ng of N-azu or 1  $\mu$ l of U-azu at pH 7.0, 37 °C for 2 h in a final volume of 10  $\mu$ L. Proteins were separated using SDS-PAGE and detected according to Materials and Methods. The apparent molecular weights of the biotinylated protein standard (M) are indicated.

neutrophil-derived azurocidin (not shown).

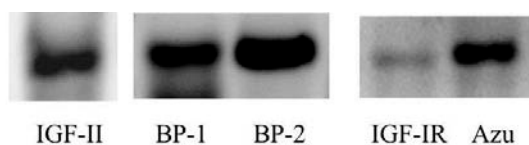
#### 4.5.2 Expression of recombinant azurocidin

We produced recombinant azurocidin by *in vitro* transcription/translation in reticulocyte lysate. The recombinant azurocidin showed no proteolytic effect on biotinylated IGFBP-1, which could be due to the lack of glycosylations. We show in paper II that proteolytically active azurocidin was glycosylated at asparagine 145. Recombinant non-glycosylated azurocidin has earlier been shown to have lower biological activity compared to glycosylated azurocidin, which suggests that glycosylation of azurocidin affects its biological activity (Iversen et al., 1999). Since transcription/translation was performed in a reticulocyte lysate, which could contain protease inhibitors, we examined the effect of the reticulocyte extract on the proteolytic activity of urine-derived azurocidin. IGFBP-1 was not cleaved by urine-derived azurocidin in the presence of the reticulocyte extract; however, the protease activity was regained after purification with heparin affinity chromatography (not shown).

#### 4.5.3 Characterisation of the IGF system in human mast cells

The protease activity used in paper I was isolated from the urine of a patient who suffered from an inflammatory skin disease. Therefore we studied the expression of azurocidin and components of the IGF-system in human mast cells. The human mast cell line HMC-1 is an immature haematopoietic cell line derived from a patient with mast cell leukemia and the only established cell line exhibiting a phenotype similar to that of human mast cells (Butterfield et al., 1988). These cells contain tryptase and produce heparin.

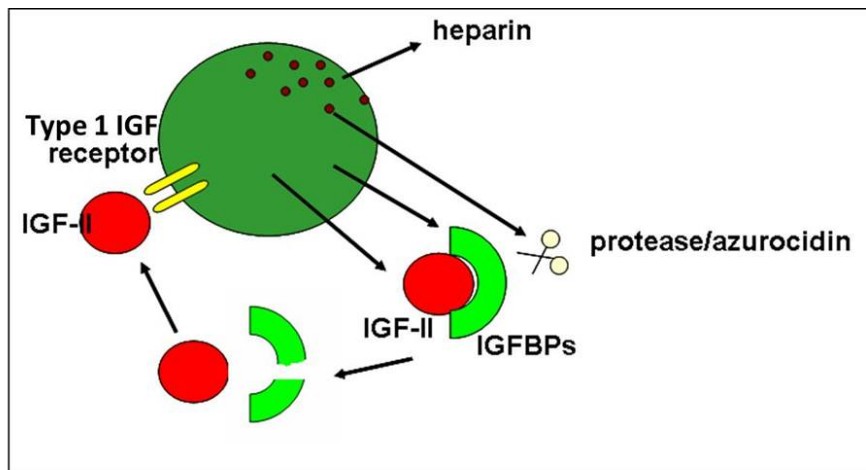
The expression of mRNA was determined by RT-PCR and HMC-1 cells were



**Figure 7. Gene expression of IGF-system components in human mast cells.**

found to express IGF-II, IGF-1R, IGFBP-1, IGFBP-2 and azurocidin (**Figure 7**). Initial studies also showed secretion of IGFBP-1, but this was not a

consistent finding and needs to be further studied. IGF-II is often up-regulated in cancer and is proposed to be an autocrine regulator in tumor cell growth. In **Figure 8** we propose a possible model for local regulation of IGFs in human mast cells.



**Figure 8. A possible model for local regulation of IGFs in mast cells.** IGFBPs are cleaved resulting in IGFBP fragments with lower affinity for IGFs and more bioactive IGFs.

When maintained in 10% fetal calf serum these cells grow in suspension culture, however, under serum-free conditions the cells adhere to plastic. In preliminary studies we found that, in the presence of the specific Type 1 IGF receptor antagonist picropodophyllin, the number of cells unattached to the culture plates was 2-fold greater than in control wells. This supports a recent study which shows that IGF-I promotes mast cell survival via activation of the phosphatidylinositol-3-kinase pathway (Lessmann et al., 2006).

#### 4.5.4 Splice variants of azurocidin

A role for splicing in regulation of azurocidin actions has not been described earlier. Unpublished data obtained in connection with studies regarding mRNA expression of azurocidin in mast cells and human leukocytes indicate that this could be the case. Three splice variants encoding peptides with the N-terminal of azurocidin followed by a short unrelated C-terminal was identified (**Figure 9**). Two of these variants contain residues 20-44 of azurocidin, which has been reported to mimic the bacterial action of azurocidin (Pereira et al., 1993). The fact that one of these splice products was found in both a mast cells and in leukocytes indicate a possible biological relevance. The protein level of azurocidin could be regulated by mRNA splicing and/or spliced azurocidin mRNA could be translated and serve some yet unknown function. It remains to be seen if these spliced mRNAs are translated into peptides in vivo.



**CAP37 P<sub>20-44</sub> NQGRHF CGGALIHARFVMTAASCFQ**

**Splice variant 1**

MGSSPLLDIVGGRKARPRQFPFLASIQ**NQGTPGLAPWCWVPMT\***

**Splice variant 2**

MGSSPLLDIVGGRKARPRQFPFLASIQ**NQGRHF CGGALIHARFVMTAASC  
FQRTLGLAPWCWVPMT\***

**Splice variant 3**

MGSSPLLDIVGGRKARPRQFPFLASIQ**NQGRHF CGGALIHARFVMTAASCFQ**SQNPGVSTVV  
LGAYDLRRRERQSRQTFSISSMSENGYDPQQNLNDLMLLQ**RFCQRDCDPRGPVSPQQRVHR  
CAHPPRWHLQWGRGHPPRLRGPGPRRGLLFP GALWPRP\***

**Figure 9. Splice variants of azurocidin detected in human mast cells and human leukocytes.** Three splice variants were found and two of these contain residues 20-44 of azurocidin, with reported antibacterial effects.

## 5 GENERAL DISCUSSION

We have observed cleavage of IGFBP-1 with the same fragmentation pattern by a novel protease-activity obtained from two different sources (urine and neutrophils) where azurocidin has been identified as the main component. Both materials have been analysed by today's available methods to exclude the presence of other proteases. Although not detected, it is possible that the partially purified material from urine and the commercially available neutrophil-derived preparation of azurocidin contain trace amounts of other serine proteases. This protease-activity was earlier shown to be inhibited by a serine-protease inhibitor (Wang et al., 2006b). It is possible that azurocidin needs to be glycosylated to be proteolytically active as the active neutrophil-derived azurocidin was glycosylated, while the recombinant non-glycosylated azurocidin was inactive.

Regardless of the source of this protease-activity, we have discovered a protease-activity which specifically cleaves IGFBP-1, -2 and -4 without degrading neither IGF-I nor IGF-II. Phylogenetic analyses of IGFBPs have shown that these three binding proteins are more closely related to each other than to the other IGFBPs (Rodgers et al., 2008). These binding-proteins are found in binary complexes with IGFs in the circulation and have the ability to leave the vascular compartment, which makes them important IGF transporters to target tissues. Besides, in contrast to other IGFBPs, IGFBP-1 and IGFBP-2 have both a C-terminal RGD-sequence and are regulated by metabolism.

Since azurocidin is released in inflammation and IGFs are important in wound healing, this protease could be an important regulator of IGF-activity in inflammation and wound healing. This thesis shows that the proteolytic IGFBP-fragments have stimulatory effects on migration and reduced inhibitory effects on IGF-induced proliferation compared to intact proteins, and that the addition of azurocidin can increase proliferation of human dermal fibroblasts.

Proteolysis has been described in the literature as the predominant way to release IGFs from IGFBPs and to increase bioactive IGFs. The regulation of IGFBP-1 appears to be more complex since we found no difference between the regulatory role of phosphorylated intact IGFBP-1 and IGFBP-1 proteolytic fragments on IGF-activity. Instead cell confluence appeared to regulate the inhibitory and potentiating effects of phosphorylated IGFBP-1.

These results are of importance for understanding the role of the IGF-system in tissue repair and could lead to the development of new treatment strategies.

## 6 CONCLUSIONS

In this thesis we have studied a novel IGFBP protease and the biological effects of the proteolytic IGFBP fragments. In summary, the conclusions of this thesis are:

1. Although IGFBP-1 fragments were detected in serum, cleavage of IGFBP-1 probably occurs at the tissue level and not in the circulation in a patient with multiple myeloma and dermatitis.
2. A neutrophil-derived preparation of azurocidin cleaves IGFBP-1, IGFBP-2 and IGFBP-4 without degrading IGFs. Phosphorylated IGFBP-1, free or bound to IGF-I is also cleaved by this preparation, while proteolysis of IGFBP-1 is reduced by IGF-II.
3. The proteolytically active preparation of neutrophil-derived azurocidin is glycosylated and determined to be 31 kDa by SDS-PAGE.
4. The same cleavage pattern of phosphorylated IGFBP-1 is obtained by incubating with either urine-derived (from patient) or neutrophil-derived azurocidin preparations.
5. Splice variants of azurocidin are expressed in human mast cells and in human leukocytes. The fact that one of these splice products was found in both cell types indicate a possible biological relevance.
6. IGFBP-1, IGFBP-2 and their proteolytic fragments stimulate migration of human dermal fibroblasts via  $\alpha 5 \beta 1$ -integrins.
7. High glucose impairs migration of human dermal fibroblasts and the addition of IGFBP-1, IGFBP-2 and their fragments increase migration in high glucose to levels observed in normal glycaemia.

8. Phosphorylated IGFBP-1 can have both potentiating and inhibitory effects on IGF-stimulated proliferation, depending on the confluence of cells.
9. IGFBP-2 fragments have reduced inhibitory effect on IGF-II action compared to intact IGFBP-2, while proteolysis of IGFBP-1 does not result in more bioactive IGF-I.
10. Neutrophil-derived azurocidin stimulates proliferation of human dermal fibroblasts.

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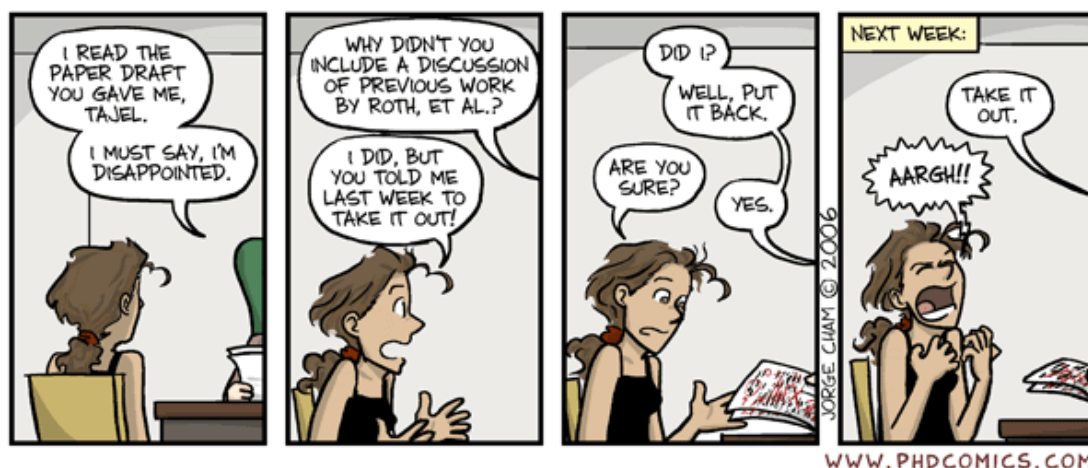
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